REMARKS

Claim 12 has been amended. Support for the amendment can be found throughout the specification, including at page 6, lines 15-19. No new matter is added by the amendments. Claims 2-3, 6-9, and 12-14 are pending in the application. Entry of the Amendment and reconsideration of the claims in view of the following Remarks is respectfully requested.

Withdrawn Rejections

In view of the Office Action's silence regarding the previous rejection of claims 2-3, 6-9, and 12-14 under 35 U.S.C. 103, as obvious over SmithKline Beecham Corp. in view of Hoifodt et al. and either Zeng et al. or Kuranami et al., Applicants understand and acknowledge that this rejection has been withdrawn.

35 U.S.C. § 112

Written Description

Claims 2, 3, 6-9 and 12-14 were rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement.

The standard for determining whether an application complies with the written description requirements of 35 U.S.C. 112, first paragraph, is whether one of ordinary skill in the art recognizes from reading the disclosure that the inventors were in possession of the claimed subject matter as of the filing date. Applicants note that there is a strong presumption that an adequate written description of the claimed invention exists (*See MPEP 2163.II.A.*), such that "a description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the Examiner to rebut the presumption." *MPEP 2163.04.* Applicants submit the Examiner has not presented evidence sufficient to rebut the presumption of adequate description.

The present claims are directed to a method for identifying genes differentially expressed between malignant cells isolated from different tissues of the same individual, and includes the steps of obtaining nearly 100% specific target cells from each of two

different tissues from the same individual by repeated immunomagnetic isolation *in vitro*, and comparing levels of mRNA expression in the target cells to identify genes differentially expressed between the target cells, in order to recognize genes possibly involved in determining metastatic characteristics of cancer cells. The Examiner asserts the claimed method encompasses the identification of previously unknown genes in virtually all life forms, but does not disclose the identification of any such genes. Applicants respectfully disagree.

Applicants note that the claims are limited to the use of malignant cells from different tissues from the same individual. The specification not only discloses, but also exemplifies, the use of embodiments of the claimed method to identify both known and unknown genes in different tissues from the same individual, that are possibly involved in determining metastatic characteristics of cancer cells. The Examiner's attention is directed to Example 1, which exemplifies the discovery of genes found to have specific expression in either primary tumor cells or axillary lymph nodes from a breast cancer patient, and which discloses that both known genes (including a cell cycle relation transcription factor and an oncogene product), as well as genes of unknown identity, were found to have specific expression (page 6, lines 15-19). Similarly, Example 2 discloses the discovery of genes exhibiting specific expression in MA-11 breast cancer cells that were injected into mice, resulting in malignant cells growing either in the leptomeninges or as spinal cord metastases. Example 2 discloses that both novel and known genes were found to have differential expression, including the novel genes LV1, LV12, and CM13 (page 7, lines 1-17). For the foregoing reasons, the specification not only discloses, but also exemplifies, the use of the claimed methods to identify novel genes having specific expression in one of two target tissues from the same individual. Consequently, the specification adequately describes the invention as claimed.

Furthermore, Applicant submit that the present claims are process claims, wherein the novelty lies in the method steps. Applicants are not claiming a previously unknown gene product. Rather, Applicants' claims are directed to a process for separating first and second tissue target cells, and identifying genes differentially expressed between them. Applicants have surprisingly discovered that the steps of the claimed methods result in

the ability to identify the specific expression of genes in malignant target cells isolated from differing tissues from the same individual. In cases involving method claims that recite a nucleic acid but wherein no particular nucleic acid is essential to the claimed invention, such that the novelty lies in the method steps, the adequacy of written description is determined with respect to the process steps, and not with respect to the nucleic acid.

The Examiner's attention is directed to Example 18 of the Revised Interim Written Description Guidelines Training Materials. In Example 18, a claim recites a method of producing a protein comprising transforming mitochondria with an expression vector comprising a nucleic acid encoding a protein of interest. *Id.* The novelty of the claim resided in the claimed method of expression in *Neurospora crassa*. No particular nucleic acid was essential to the claimed invention; the claim was directed to a nucleic acid encoding any protein of interest. *Id.* The guidelines conclude that the claimed invention is adequately described, even though the specification only disclosed the expression of a single nucleic acid. *Id.*

The forgoing Example is applicable to the present case. The instant claims are not directed to a gene product, nor do they require the identification of any particular gene product. Rather, the claims are directed to a novel process for identifying genes differentially expressed between first and second tissue target cells obtained by the recited immunomagnetic isolation steps. Therefore, the claimed method is not limited to any particular gene or genes; but is applicable to the identification of any genes that are differentially expressed. Applicants submit, therefore, that the claims are adequately described for this additional reason.

Applicants submit that the claims are adequately described for at least the foregoing reasons. Withdrawal of the rejection is respectfully requested.

Enablement

Claims 2, 3, 6-9 and 12-14 were rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. Applicants traverse this rejection.

The Examiner "has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention." *MPEP 2164.04*. A finding that further experimentation is necessary to practice an invention is insufficient to question the enablement of the claims. "The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation." *MPEP 2164.01*. In light of the foregoing guidelines, the present claims are clearly enabled by the specification.

The Examiner asserts the disclosure fails to disclose the identification of any previously unknown genes, what these genes are, or what these genes could be. Applicants disagree. As discussed above regarding the Written Description rejection, the specification does in fact disclose and exemplify, in Examples 1 and 2, the identification of multiple novel genes that are differentially expressed between the first and second target tissues as claimed.

Furthermore, and contrary to the Examiner's assertions, the specification also discloses what the identified novel genes are or could be. The disclosure teaches that many types of cancer show typical tissue-preferenced spread to other regions of the body (page 1, lines 8-10), and that the ability of tumor cells to spread preferentially to certain areas is associated with the expression of specific proteins that may be involved in, for example, enabling the tumor cells to home to the target organ, move to and invade the host tissue, respond to local growth factors, or induce angiogenesis (lines 16-21). The specification discloses embodiments of the present invention that provide a method for detecting genes with site-specific expression patterns in tumor cells residing in different tissues, and discloses that the method can identify the genes encoding the proteins responsible for the tissue-preferenced cancer spread (page 1, lines 4-6; page 4, lines 4-6 and 11-13).

Furthermore, Example 1 specifically identifies cell cycle related transcription factor and known oncogene products as genes that were identified by an embodiment of the present invention (page 6, lines 19-20). Consequently, the specification clearly discloses the nature of the genes that may be identified by the claimed methods, and

provides working examples that resulted in the identification of multiple genes. Therefore, one of skill in the art could readily make and use the presently claimed invention. As a result, Applicants respectfully submit the Examiner has not met the initial burden necessary to question the enablement of the claims.

The Examiner also contends, however, that the claims lack enablement due to the alleged non-disclosure of starting materials and reaction conditions. Applicants disagree. In order to render claims enabled, the specification "need not disclose what is well-known to those skilled in the art and **preferably omits** that which is well-known to those skilled and already available to the public." *MPEP 2164.05(a)*(emphasis added). The specification discloses multiple references that describe in detail techniques that can be used for carrying out the individual steps of the invention (*See, e.g.*, page 4, lines 25-27; page 5, lines 6-8; page 6, lines 9-10).

In response, the Examiner asserts the references cited by the specification are not properly incorporated by reference, and were not considered with respect to any 35 U.S.C. 112, first paragraph rejections. Applicants respectfully submit that the Examiner's refusal to consider the citations is erroneous. The cited references point to techniques well-known in the art. As stated above, the specification need not disclose what is well-known in the art. Therefore, the cited references are relevant in establishing the adequacy of description, regardless of whether or not they have been properly incorporated by reference.

The Examiner, however, cites to *Genentech v. Novo Nordish A/S* for the proposition that a specification's failure to disclose specific starting materials or reaction conditions renders the disclosure non-enabling. Applicants submit, however, that *Genentech* is not applicable to the present case. The claims at issue in *Genentech* were directed to a method of making hGH using cleavable protein expression (*Genentech v. Novo Nordish A/S*, 42 USPQ2d 1001). The disclosure provided not a single working example of making hGH using the claimed method. The court held that the claims were nonenabled, since "the specification, not the knowledge of one skilled in the art, must supply the novel aspects of an invention in order to constitute adequate enablement." *Id.*

at 1005 (emphasis added). The court based its holding on the fact that: 1) the specification claimed to solve an unresolved problem of an earlier application, yet comprised no additional disclosure relative to the earlier application *Id.*; 2) the existence of evidence in the prior art showing that the teachings of the disclosure would not work well in the claimed method; and 3) evidence that no one had ever made a human protein via cleavable fusion expression, such that the technology was unpredictable and in an early stage of development. *Id.* at 1005-06.

In the present case, by contrast, the novelty of the claimed invention is not dependent upon the specific techniques that can be used in the recited steps of immunomagnetic isolation, determination of levels of mRNA expression, or comparing levels of mRNA expression. As stated above, techniques that can be used in each of these steps individually are well-known in the prior art. Therefore, unlike the fact pattern of *Genentech*, the specification does not fail to describe the novel aspects of the claimed invention. Previously, it was not thought possible to perform meaningful gene cloning experiments on specimens of solid tumors and metastases, and on malignant cells in blood and bone marrow, with the object of identifying genes with site-specific expression (page 3, line 12 to page 4, line 2). The present specification surprisingly discloses, however, that a method comprising the novel claimed combination of steps provides the ability to identify genes that may be involved in determining the metastatic characteristics of cancer cells.

Moreover, and also unlike the fact pattern of *Genentech*, the present specification exemplifies that the invention can in fact successfully identify differentially expressed genes as claimed. The Examiner has presented no evidence that Applicants' invention as disclosed does not work, or that the specific techniques useful in each step of the claims relate to unpredictable technologies in an early stage of development. Indeed, as discussed above, techniques for use in the individual steps of the claims are well known in the art.

Furthermore, Applicants submit that even if the specification's citation of references is ignored, and even if the alleged lack of disclosure was sufficient to establish

a reasonable basis to question enablement, Applicants have presented rebuttal evidence in the form of a Declaration under 37 C.F.R. 1.132 that is sufficient to overcome the rejection.

The present claims include the steps of: 1) immunomagnetically isolating cells *in vitro* from different tissues, 2) determining levels of mRNA in the cells; and 3) comparing levels of mRNA between cells to identify whether the mRNA is differentially expressed. In the Declaration under 37 C.F.R. 1.132 submitted on July 26, 2002, Applicants provided convincing proof that techniques for performing each of these steps individually were well-known in the art at the time of filing of the present invention, such that one of skill could readily make and use the claimed invention in light of the specification, when combined with the teachings of the prior art. A Declaration under 1.132 that cites references to show what one of skill in the art knew at the time of filing of the application is evidence that must be considered. *MPEP 2164.05*. The Examiner is reminded that the evidence provided in the Declaration need not be conclusive, but merely convincing to one skilled in the art. *Id.*

The Declaration explicitly states that a scientist in the field of molecular and cellular biology would have known how to perform individually each of the foregoing method steps (*See* page 2 of the Declaration). The Declaration additionally provides specific references demonstrating that one of skill in the art, from reading the specification, would have been able to practice the method steps as claimed using knowledge that is both well-known and readily available in the prior art (*See* page 2, last full paragraph; page 3, second and third paragraphs; page 5, last paragraph; and page 7, last paragraph). The Declarant also provided three scientific references demonstrating the use of the present invention to successfully isolate nucleic acid sequences potentially involved in metastasis (*See* page 8). Applicants submit, therefore, that Applicants have overcome any reasonable basis to question the enablement of the claims.

Additionally, Applicants submit in an Appendix to this Response four additional, post-filing date references demonstrating in detail the use of the claimed invention to successfully identify genes with differential expression between primary and metastatic

tumors. Although post-filing date evidence cannot render an insufficient disclosure enabling, it may be used to prove that a disclosure is enabling, and to substantiate the accuracy of statements made in the specification. *In re Brana*, 34 USPQ2d 1437, 1441 n.19 (Fed. Cir. 1995)(citing *In re Marzocchi*, 169 USPQ 367, 370 n.4 (CCPA 1971)). Ree et al. (*Cancer Res* 59:4675-80 (1990)) and Bratland et al. (*Cancer Res.* 60:5578-83 (2000)) disclose the use of the claimed invention to identify differentially expressed genes and gene products that may mediate the growth response of tumor cells upon establishment in a secondary organ, including a known intracellular effector molecule, and a novel gene product that appeared to distinguish breast cancer cells with metastatic potential from cells without metastatic potential (*See* Abtract). Ree et al. (*Int. J. Cancer*, 97:28-33 (2002)), and Rec et al. (*Anticancer Res.* 22(4), 1949-57 (2002)) provides detailed information on how to combine an immunomagnetic selection procedure and differential display analysis to reveal gene expression profiles that may characterize red bone marrow micrometastatic cells. Applicants submit that the foregoing references substantiate the accuracy of the specification's assertion of enablement of the claimed methods.

For at least the foregoing reasons, Applicants submit that claims 2, 3, 6-9 and 12-14 are fully enabled by the specification. Withdrawal of the rejection is requested.

35 U.S.C. § 101

Claims 2, 3, 6-9, and 12-14 were rejected under 35 U.S.C.101 for lacking either a specific and substantial utility, or a well-established utility. The Examiner states that the claimed methods result in the identification of "previously unknown genes," but that the genes are not defined as having any specific or substantial utility. Applicants traverse this rejection, and submit that the claims possess both a specific and substantial utility and a well-established utility.

The specification discloses that the identification of genes expressing proteins associated with the ability of tumors to preferentially metastasize to other tissues is of great importance for diagnosis and therapy (page 1, lines 21-23). Example 2 discloses an embodiment of the invention leading to the identification of LV12, a novel gene inversely

correlated to progression and metastasis of breast cancer cells, such that low expression of LV12 was related to short survival of the patients (page 7, lines 9-13). Therefore, the specification discloses that genes identified by the present methods can have the specific and substantial utility of diagnosing the prognosis of cancer patients.

Furthermore, the present invention provides for easier identification of genes preferentially expressed between primary tumors and metastatic tumors (Declaration at page 2). Consequently, one skilled in the art would immediately recognize that genes identified using the claimed methods can be useful as diagnostic markers of the metastatic potential of cancerous cells. One of skill would also recognize that such a use is specific, substantial, and credible. Therefore, the present claims also possess a well-established utility. *MPEP 2107.02 II.B*. The Examiner's attention is directed to Example 12 of the *Revised Interim Utility Guidelines Training Materials*, where the disclosure that a receptor "is present on the cell membranes of melanoma cells, but not on the cell membranes of normal skin cells," combined with the knowledge that it would be desirable "to selectively detect melanoma cells so as to diagnose that type of cancer," was declared sufficient to provide a well-established utility.

For at least the foregoing reasons, Applicants submit that claims 2, 3, 6-9, and 12-14 possess the utility required by 35 U.S.C. 101. Withdrawal of the rejection is requested.

Conclusion

In view of the above amendments and remarks, Applicant respectfully requests a Notice of Allowance. If the Examiner believes a telephone conference would advance the prosecution of this application, the Examiner is invited to telephone the undersigned at the below-listed telephone number.

Respectfully submitted,

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[CANCER RESEARCH 59, 3671–3677, September 15, 1999]

Expression of a Novel Factor in Human Breast Cancer Cells with Metastatic Potential[1]

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ABSTRACT

Clinical and experimental evidence suggests that tumor cells shed into the circulation from solid cancers are ineffective in forming distant metastasis unless the cells are able to respond to growth conditions offered by the secondary organs. To identify the phenotypic properties that are specific for such growth response, we injected carcinoma cells, which had been recovered from bone marrow micrometastases in a breast cancer patient who was clinically devoid of overt metastatic disease and established in culture, into the systemic circulation of immunodeficient rats. The animals developed metastases in the central nervous system, and metastatic tumor cells were isolated with immunomagnetic beads coated with an antibody that was reactive with human cells. The segregated cell population was compared with the injected cells by means of differential display analysis, and two candidate fragments were identified as up-regulated in the fully metastatic cells. The first was an intracellular effector molecule involved in tyrosine kinase signaling, known to mediate nerve growth factor-dependent promotion of cell survival. The second was a novel gene product (termed candidate of metastasis-1), presumably encoding a DNA-binding protein of helix-turn-helix type. Constitutive expression of *candidate of metastasis-1* seemed to distinguish breast cancer cells with metastatic potential from cells without metastatic potential. Hence, our experimental approach identified factors that may mediate the growth response of tumor cells upon establishment in a secondary organ and, thereby, contribute to the metastatic phenotype.

INTRODUCTION

Recent experimental evidence implies that the ability of tumor cells to initiate growth after establishment in the secondary organ and to continue subsequent growth from micrometastases into macroscopic tumors is among the primary determinants of metastasis formation (1, 2). Early in the chain of tumor progression, metastases are limited in size and location (*e.g.*, bone marrow micrometastasis) because the facility for metastatic growth has not yet been fully developed (3). The red bone marrow compartment still represents an important indicator organ of hematogenous micrometastatic spread in several types of carcinomas (4). However, disseminated tumor cells detected in the bone marrow of patients with early stages of solid cancers may simply represent irrelevant shed cells unless they hold biological properties that are crucial for establishment within secondary organs (5, 6).

Solid cancers metastasize in a selective manner to distant organs, and the organ specificity of the metastatic process is assumed to be governed by interactions between the malignant cells and local microenvironment factors (7–10). The lack of biologically relevant model systems to study tumor-host interactions in metastasis, however, has limited insight into which molecular mechanisms are involved in the complex regulatory events of this fundamental aspect of cancer biology (11, 12).

The aim of this study was to identify cellular factors participating in the response of breast carcinoma cells to growth conditions offered by the target organ upon metastasis formation. Carcinoma cells in culture, originating from bone marrow micrometastases in a breast cancer patient who was clinically devoid of overt metastatic disease, were injected into the systemic circulation of immunodeficient rats, which subsequently developed metastases in the CNS.[3] The carcinoma cells from the metastases were isolated by immunomagnetic selection and compared with the injected cells by means of differential display analysis, revealing a novel factor induced in the metastatic cells. We assume that this factor may mediate the growth response of tumor cells upon establishment in a secondary organ and, thereby, contribute to a cellular phenotype that is appropriate for cancer metastasis.

MATERIALS AND METHODS

Experimental CNS Metastasis Model. Micrometastatic cells were isolated by immunomagnetic selection of a bone marrow sample taken from a breast cancer patient who had invasive lobular carcinoma but who was clinically devoid of metastatic disease. The isolated carcinoma cells were established as the MA-11 cell line, as reported previously (13). The MA-11 cells were passaged in monolayer cultures for >100 passages before they were used in these animal experiments. We developed a novel experimental model for breast cancer metastasis to the CNS, using different routes of injection of MA-11 cells in athymic nude rats (Han: *rnu/rnu* Rowett), bred in the nude rodent facility at the Norwegian Radium Hospital (14). Cells in exponential growth phase were harvested, and suspensions were prepared for injection into either the LV (2.5×10^6 cells in 200 μl per rat) or the CM (2.5×10^4 cells in 30 μl per rat). The rats were sacrificed immediately upon symptoms of CNS disease. The care of animals and the experimental protocol were reviewed and approved by the National Animal Research Authority and carried out according to the European Convention for the Protection of Vertebrates Used for Scientific Purposes.

Immunomagnetic Cell Preparation. The affected tissues (spinal cords of rats injected in the LV and meninges of rats injected in the CM) were carefully removed and dissected. Tissue preparations from two to three equivalent rats were pooled and treated by mechanical dissociation to obtain cell suspensions (3.5×10^6–5.0×10^6 cells in 1 ml) for immunomagnetic cell separation. All solutions and cell preparations were kept on ice during the whole procedure to avoid nonspecific binding of immunobeads. A monoclonal antibody had previously been developed by immunizing nude mice (BALB/c *nu/nu*) with fresh tumor cells from an unclassified soft tissue sarcoma of high-grade malignancy (15). The antibody revealed a unique panhuman reactivity when tested by immunostaining on a wide range of human tumor cells, normal fibroblasts, and mononuclear cells from peripheral blood and bone marrow. No cell surface binding was observed on cell lines derived from rodents, and no reactivity was shown with normal tissue sections from mice, rats, or dogs.[4] This antibody, purified from murine ascites, was conjugated to superparamagnetic sheep-

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[3] The abbreviations used are: CNS, central nervous system; LV, left cardiac ventricle; CM, subarachnoid space "cisterna magna"; comt, candidate of metastasis-1; HTH, helix-turn-helix motif; hom, *Drosophila antennapedia* homeodomain; hnf, hnf-3/γ fork protein; pou, pit cts domain; fia, factor for inversion stimulation; ER, estrogen receptor.

[4] Unpublished data.

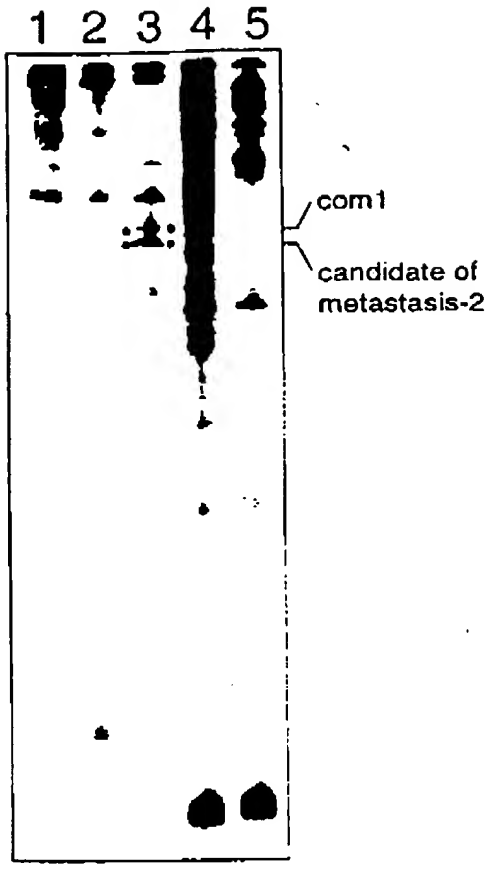

Fig. 2. Differential display analysis of MA-11 cell populations. Candidate PCR fragments (*com1* and *candidate of metastasis-2*) are indicated. *Lane 1*, cell culture population (representing micrometastatic cells in bone marrow); *Lane 2*, CM population (cells isolated from meningeal tumors); *Lane 3*, LV population (cells isolated from spinal cord metastases); *Lane 4*, rat meninges (negative selection control for the CM population); *Lane 5*, rat spinal cord (negative selection control for the LV population). Primers used were 5'-T_{11}VA-3' and AP2 (5'-GAAACGGGAC-3'). Candidate of metastasis-2 turned out to represent a false positive fragment.

actively proliferating tumors. Small tumors, presumably nonsymptomatic, were found also in the brain parenchyma of the rats injected LV (data not shown).

Metastatic MA-11 cells isolated by immunomagnetic separation of the tumor-affected tissues were examined by light microscopy, and the selected cell fractions were evaluated for host cell contamination (*i.e.*, presence of cells with fewer than five immunobeads bound to their surface). This revealed a highly enriched CM population, whereas the fraction isolated from the intramedullary spinal cord tumors (LV population) contained ~20% cells defined as contaminants (data not shown).

To identify site-specific gene expression, we compared the metastatic cells (selected LV population) with the CNS growth control (selected CM population) as well as to the injected cells (MA-11 cells in culture) by means of differential display analysis. Differentially expressed PCR fragments were considered to represent true metastatic properties if they were present in the LV population and absent in the CM population and MA-11 cells in culture and they were not concomitantly present in rat spinal cord (*i.e.*, not evidently representing contamination from host tissue). Of the candidate fragments, differential expression was confirmed by Northern blot hybridization for two bands. The first, appearing to be the Grb2-associated binder-1 (26, 27), showed ~2-fold higher levels in the LV population compared with the CM population, which further showed another ~2-fold up-regulation relative to the MA-11 cells in culture (data not shown). The second, a novel "candidate of metastasis" (com1; Fig. 2), revealed ~3-fold higher expression in the LV population compared with MA-11 cells in culture and ~2-fold increase compared with the CNS growth control (CM population), as well as a complete lack of expression by the host tissues (Fig. 3).

The com1 PCR fragment matched expressed sequence tags found in several sequence databases. Because these expressed sequence tags revealed some slight differences in their sequences, the missing 5' end of *com1* cDNA was amplified from cDNA generated from nonlactating mammary gland mRNA, which was considered to represent a "common lineage" phenotype of breast gland-derived neoplasms. The 5' fragment contained the complete coding sequence, a finding consistent with the nature of PCR products identified by differential display analysis, which generates 3'-nontranslated expressed sequence tags.

The full-length *com1* cDNA sequence encoding an 82 amino acid peptide was determined (Fig. 4) and appeared to be the human homologue of the rat *p8* cDNA (18). Protein segment analysis (Fig. 5) indicated that com1 shows ~70% sequence identity with the rat p8 protein and lower but still significant similarity with H_{R-2}, H_{R-1}, and H_R (the three key helices) of four types of HTH DNA-binding domains: hom (19), bnf (20), pue (21), and fia (22).

To examine whether com1 expression may distinguish breast cancer cells with metastatic potential from cells without, we analyzed a

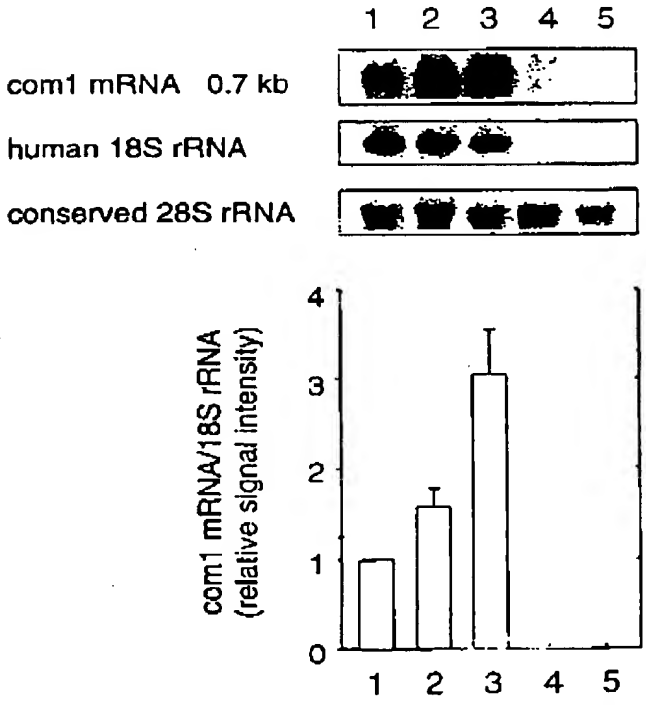

Fig. 3. Differential expression of *com1* mRNA. *Lanes 1–5* as in Fig. 2. The level of *com1* mRNA was analyzed by Northern blot hybridization [illegible] 18S rRNA was measured as a phenotypic control, whereas the 28S rRNA was examined as an assay control. *Bottom, columns*, mean Phosphoimager densitometry values [illegible] of the com mRNA/18S rRNA signals, relative to the value of MA-11 cells in culture. SD

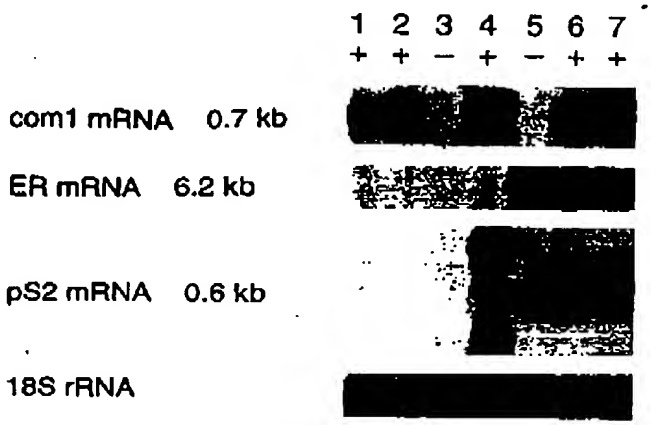

Fig. 6. Expression of *com1* mRNA in breast cancer cell lines. Cell lines shown to be experimentally metastatic (+) or not (−) were analyzed by Northern blot hybridization. *Lane 1*, MA-11; *Lane 2*, MT-1; *Lane 3*, MDA-MB-231; *Lane 4*, MDA-MB-435; *Lane 5*, MCF-7; *Lane 6*, MCF7/LCC1; and *Lane 7*, MCF7/LCC2. Expression of mRNAs for *ER* and *pS2* was measured as phenotypic controls. The 18S rRNA was estimated as an assay control.

+hQNSxR+ (where + denotes positively charged residue and h denotes a hydrophobic residue). In pue, a winged HTH motif with a small β-sheet located between H_{R-2} and H_{R-1}, the latter helix shows similarity with the corresponding region of com1 and rat p8. The conserved sequence pattern is ++EAxA. In fla, an HTH belonging to the toxin repressor subfamily, the similarity to com1 and rat p8 is essentially located in H_{R-3}, represented by the pattern DxxDLYxL.

The cultured MA-11 cells were com1 positive, suggesting a requirement of constitutive com1 expression in carcinoma cells from which metastatic tumors originate. Hence, it is conceivable that com1 expression may distinguish breast cancer cells with facility to develop a fully metastatic potential from cells without. This notion was supported by *com1* expression observed exclusively in breast cancer cell lines that are experimentally metastatic. The human MT-1 cell line (34) forms bone-eroding metastases in the spine upon LV injection in rats (14). The MDA-MB-435 cell line cultured in our laboratory gives rise to extensive metastasis when inoculated into rodents, whereas our MDA-MB-231 cell line does not metastasize, whatever routes of injection tested.[4] The MCF-7 cell line and the derivative sublines MCF7/LCC1 and MCF7/LCC2 reflect the phenotypes of carcinoma cells observed during clinical progression of breast cancer. The parental cells are highly responsive to estrogens but poorly metastatic in animal models (35). The estrogen-independent MCF7/LCC1 cells possess a metastatic phenotype but are still ER positive (25, 35). The MCF7/LCC2 cells have retained the phenotype of the MCF7/LCC1 cells and are also tamoxifen resistant (30).

The Grb2-associated binder-1, identified as an intracellular effector molecule in tyrosine kinase signaling (26, 27), is shown to mediate nerve growth factor-dependent inhibition of apoptosis in promotion of cell survival (36). Our data support the concept that responses mediated by receptor tyrosine kinases are crucial for establishment of CNS metastasis (37), irrespective of the mechanism of such tumor growth (by direct extension as for meningeal metastasis or by hematogenous dissemination when parenchymal metastases are formed; Refs. 31 and 38).

Metastasis in the CNS represents a severe complication for patients with breast cancer. After clinical diagnosis of such advanced cancer disease, survival is limited (39). Breast cancer metastasis to the CNS or other distant organs may appear after extended periods of apparent freedom from disease (3, 39), a phenomenon that is conceptually due to a somewhat loosely defined proliferative reactivation of disseminated tumor cells (1, 2, 10). This may also explain why dormant micrometastases escape conventional therapies that are directed specifically against actively dividing tumor cells (40).

In conclusion, this study has identified and partially characterized a novel factor, termed com1, expressed in breast carcinoma cells forming metastatic tumors. com1 presumably represents a DNA-binding protein of HTH type, which might participate in intracellular signaling mediating growth responses of the tumor cells upon establishment in a secondary organ. Future studies of com1 function will include the identification of growth factors that induce com1 expression as well as presumably com1-responsive genes to map the complete regulatory pathway involved in this process.

ACKNOWLEDGMENTS

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Expression of a Novel Factor, com1, Is Regulated by 1,25-Dihydroxyvitamin D_3 in Breast Cancer Cells[1]

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ABSTRACT

Tumor cells and their surrounding microenvironment produce a variety of factors that promote tumor growth and metastasis. We recently identified a nuclear factor, termed com1, that is up-regulated in human breast carcinoma cells on formation of experimental metastatic tumors and is assumed to act as a growth-promoting factor in breast cancer. 1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] is a potent inhibitor of growth in breast cancer both *in vitro* and *in vivo*. We compared the growth-regulatory mechanisms of nontumorigenic and estrogen-dependent MCF-7 cells with those of the tumorigenic and tamoxifen-resistant subline MCF7/LCC2 in the presence of 1,25$(OH)_2D_3$. Proliferation of MCF7/LCC2 cells, which revealed constitutive com1 expression, was inhibited by 1,25$(OH)_2D_3$ (10^{-7} M). This was strongly associated with cell cycle arrest in G_1 phase, consistent with accumulation of the hypophosphorylated form of the retinoblastoma protein as well as the induction of the cyclin-dependent kinase inhibitor p21. These cell cycle events were preceded by a transient up-regulation (5–8-fold) of *com1* mRNA. Furthermore, clonal growth of the MCF7/LCC2 cells was also inhibited by 1,25$(OH)_2D_3$ (10^{-7} M), and when the com1-negative MCF-7 cells were stably transfected with com1, the resulting MCF7/com1 cells showed a significant decrease in colony formation. These results seem to indicate that rather than promoting growth, com1 may participate in the regulatory pathway involved in cellular growth inhibition when recruited by inhibitory signals.

INTRODUCTION

After malignant transformation, tumor cells and their surrounding stroma produce a variety of factors within the tumor cell environment to promote tumor growth and metastasis (1). Moreover, current evidence suggests that metastasis formation is primarily the result of the ability of disseminated tumor cells to initiate and continue growth in the target organ (2, 3). We recently reported a novel approach, comparing the phenotypes of human breast carcinoma cells acquired from an early step in clinical tumor progression and the cell population isolated from experimental metastases formed by these tumor cells, to identify properties that might prevail in the metastatic cells. A factor termed com1 was identified as up-regulated in the metastatic cell population. com1 presumably represents a helix-turn-helix-type DNA-binding protein and may participate in the response of breast carcinoma cells to growth conditions offered by the target organ on formation of distant metastases (4).

In addition to its role in the calcium metabolism, vitamin D also promotes tissue differentiation and inhibits cellular proliferation. Several reports have described antiproliferative effects of vitamin D in breast cancer cells in culture (5–7), and it has been suggested that noncalcemic vitamin D analogues may have a clinical potential in the treatment of breast cancer (8, 9). Furthermore, epidemiological studies have shown an inverse correlation between average annual sunlight exposure and the incidence of breast cancer, suggesting an association between endogenous vitamin D production and breast carcinogenesis (10, 11).

The aim of the present study was to examine whether cellular com1 expression may be associated with vitamin D-dependent growth regulation of breast cancer cells. We used the human cell line MCF-7 and its derivative subline MCF7/LCC2, which reflect phenotypes of the carcinoma cells observed during clinical progression of breast cancer. The com1-negative MCF-7 cells (4) are highly responsive to estrogens but poorly tumorigenic in animal models (12). In contrast, the estrogen-independent MCF7/LCC2 cells, which are also tamoxifen resistant and possess a tumorigenic phenotype (12, 13), show constitutive com1 expression (4). The active metabolite of vitamin D, 1,25$(OH)_2D_3$,[3] promoted growth inhibition of the MCF7/LCC2 cells mediated by induction of the cdk inhibitor p21 and concomitant hypophosphorylation of pRB. These events, ultimately leading to cell cycle arrest in the G_1 phase, were preceded by a transient induction of cellular com1 expression. Because expression of *com1* mRNA was also inversely correlated to clonal growth of MCF-7 cell lines, we postulate that com1 may participate in the regulatory pathway involved in cellular growth control when recruited by inhibitory signals.

MATERIALS AND METHODS

Cell Cultures. The MCF-7 human breast cancer cells were routinely grown in MEM containing phenol red (Life Technologies, Inc., Rockville, MD) supplemented with 5% FCS (Life Technologies, Inc.), insulin (5.0 μM; Sigma, St. Louis, MO), and glutamine (2.0 mM; Life Technologies, Inc.). The subline MCF7/LCC2 was routinely grown in MEM without phenol red (Life Technologies, Inc.) supplemented with insulin and glutamine as described above, in addition to 5% steroid-depleted FCS. The serum was stripped of endogenous steroids by treatment with charcoal as described previously (14). The MCF7/com1 and MCF7/pMAM cells were grown in the same medium as MCF-7 supplemented with Geneticin (G418; Sigma). Cell cultures were kept at 37°C in a humidified 5% CO_2 atmosphere and refed every 3–4 days. Twenty-four h before the start of all experiments, media were exchanged, and cells were further incubated in the absence of insulin (defined as experimental media). All experiments were conducted on cells in exponential growth phase.

Cellular Proliferation. The MCF-7 and MCF7/LCC2 cells were seeded in experimental media on culture dishes (6-cm in diameter; 2.5×10^5 cells/dish). After 24 h (at the start of the experimental period), medium was changed, and the cells were grown in the absence or presence of 1,25$(OH)_2D_3$ (10^{-7} M; a generous gift from Dr. L. Binderup; Leo Pharmaceutical Products, Ballerup, Denmark) for up to an additional 96 h. For both cell lines, the seeding of this particular cell number resulted in near confluent growth at the end of the

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[3] The abbreviations used are: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; cyclin-dependent kinase; pRB, retinoblastoma protein; RA, [illegible] 20

incubation period. At time points 0, 24, 48, 72, and 96 h, respectively, control and treated cells were harvested by trypsination, and trypan blue-excluding cells were counted using a Bürker's counting chamber. All cell number determinations were performed in triplicate, and the internal variation in counts was <5%. Three separate sets of experiments with three parallel samples for each condition were performed.

Flow Cytometry Analysis. Cells were harvested, fixed in 1% paraformaldehyde, and subsequently resuspended in 100% methanol for storage at −20°C. The staining procedure was performed for each sample in a 50-μl solution consisting of 5 units of biotinylated terminal transferase (Boehringer Mannheim, Mannheim, Germany), 0.5 nmol of biotin-16-dUTP (Boehringer Mannheim), 1.5 mM $CoCl_2$, and 0.1 mM DTT. After incubation for 30 min at 37°C, cells were washed with PBS and incubated for another 30 min in 50 μl of streptavidin-conjugated FITC (Amersham Pharmacia Biotech, Uppsala, Sweden) diluted 1:50 in PBS with 0.1% Triton X-100. Cell pellets were finally resuspended in 500 μl of PBS containing 0.1% Triton X-100, 100 μg/ml RNase A, and 5 μg/ml propidium iodide. Stained cells were analyzed in a FACStar+ laser flow cytometer with excitation at 488 nm, and DNA content (integrated propidium iodide fluorescence, collected using linear amplification) *versus* replicative cell fractions (FITC fluorescence intensity, collected using logarithmic amplification) was measured.

Western Blot Analysis. Cells were harvested and homogenized in ice-cold lysis buffer [250 mM NaCl, 2 mM EDTA, 0.1% NP40, 1 mM DTT, 1 mM NaF, 1 mM orthovanadate, 60 mM β-glycerophosphate, 50 μg/ml phenylmethylsulfonyl fluoride, and 2 μg/ml each of aprotinin and leupeptin (pH 7.2); Ref. 15)]. Aliquots of 30 μg of total protein were separated on 7.5% (for detection of pRB) or 12% (for detection of the other proteins) SDS-PAGE and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech) by standard methods. The membranes were blocked in TBS-T containing 5% nonfat dry milk for 1 h at room temperature and subsequently incubated with 1 μg/ml dilutions of mouse antihuman antibodies in TBS-T for 2 h at room temperature. The antibodies were anti-pRB [G3–254; purchased from PharMingen (San Diego, CA)] and anti-p21 (C-19), anti-p27 (C-19), anti-cdk4 (C-22), and anti-cyclin D1 C-20 [all obtained from Santa Cruz Biotechnology (Santa Cruz, CA)]. After four washes with TBS-T, the membranes were incubated with a 1:6000 dilution of horseradish peroxidase-linked secondary antibody (Bio-Rad, Hercules, CA), and immunoreactive proteins were visualized with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Generation of Stable MCF7/com1 and MCF7/pMAM Transfectants. A PCR-generated DNA fragment containing the complete coding region of *com1* (4) was ligated in-frame into the *EcoRI* site behind the Dex-inducible promoter of the mammalian expression vector pMAMneo (Clontech, Hampshire, United Kingdom). This construct was transfected into MCF-7 cells by electroporation. Individual stable transfectants were selected in media containing 2 mg/ml G418 and maintained in media containing 500 μg/ml G418. Control MCF7/pMAM transfectants were generated correspondingly, but with the use of an empty pMAMneo vector instead. The com1 transcript was found to be inducible by Dex in the MCF7/com1 cells.

Northern Blot Analysis. Total RNA was extracted and analyzed by standard Northern blotting techniques. Samples of 10 μg of RNA were resolved by gel electrophoresis before transfer onto Hybond-N+ membranes (Amersham Pharmacia Biotech). The cDNA probes were labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) by using the random priming technique, and standard Church hybridization conditions were used. To evaluate the amounts of RNA loaded, the filters were rehybridized to a kinase-labeled oligonucleotide probe complementary to nucleotides 287–305 of human 18S rRNA. Finally, the autoradiographs were subjected to densitometric measurements in a Molecular Dynamics 300A laser densitometer, and the mRNA expression levels relative to 18S rRNA were calculated.

Clonal Growth Assay. Soft agar cultures were performed in tubes by adding 0.2 ml of nude rat blood diluted 1:8 in experimental media, 0.6 ml of 0.5% agar (Difco Laboratories, Ltd., Surrey, United Kingdom) in experimental media, and a 0.2-ml suspension of cells grown for 24 h in experimental media (500 cells/culture sample). The cultures contained a final concentration of 5% steroid-depleted FCS for the MCF7/LCC2 cells or 15% FCS for the MCF-7, MCF7/pMAM, and MCF7/com1 cells. The tubes were incubated at 37°C in 5% CO_2, 5% O_2, and 90% N_2. Experimental media (1 ml) with the proper concentrations of $1,25(OH)_2D_3$, Dex, or ethanol vehicle were added on days 7 and 14. Colonies of >2 mm were scored after 21 days of incubation using a Nikon stereomicroscope. Experiments with six to nine parallel samples for each condition were performed.

Statistics. The statistical analyses were performed using the SigmaStat software program (Jandel, Erkrath, Germany) with a significance level of $P < 0.05$.

RESULTS

Regulation of Cellular Proliferation and the Cell Cycle by $1,25(OH)_2D_3$. The MCF-7 and MCF7/LCC2 cells were cultured in the absence or presence of $1,25(OH)_2D_3$ (10^{-7} M), and growth was measured after 0–96 h of incubation (Fig. 1). Both cell lines revealed an exponential growth pattern of untreated cells (a 6–8-fold increase in cell number after 96 h), whereas a striking difference was observed between the cell lines in the presence of $1,25(OH)_2D_3$. The MCF-7 cells still showed exponential growth, although the growth rate was significantly reduced after 72–96 h of treatment. In the MCF7/LCC2 cells, the exponential growth pattern was abolished in the presence of $1,25(OH)_2D_3$. However, a significant inhibitory effect compared with the control situation was not observed before 72 h.

To characterize the mechanism of growth inhibition by $1,25(OH)_2D_3$, the cells were cultured in the absence or presence of $1,25(OH)_2D_3$ (10^{-7} M) for 48 h. First, cell cycle profiles were analyzed by flow cytometry (Fig. 2). The cell cycle distribution showed $1,25(OH)_2D_3$-dependent accumulation of MCF7/LCC2 cells in the G_1 phase. Signals corresponding to apoptotic cells were almost absent, as shown by gates R2 (displaying apoptotic cell fractions) of the histograms. Subsequently, the phosphorylation status of pRB was examined (Fig. 3). Consistent with the flow cytometry data, an accumulation of the hypophosphorylated form of pRB was observed in the $1,25(OH)_2D_3$-treated MCF7/LCC2 cells.

Among the factors involved in regulation of the cell cycle, *p21* has been shown to be a primary target for transcriptional regulation by vitamin D (9, 16). Hence, mRNA expression of *p21* and other factors involved in regulation of the G_1 phase was analyzed (Fig. 4*a*). Both cell lines showed constitutive mRNA expression of the cdk inhibitors

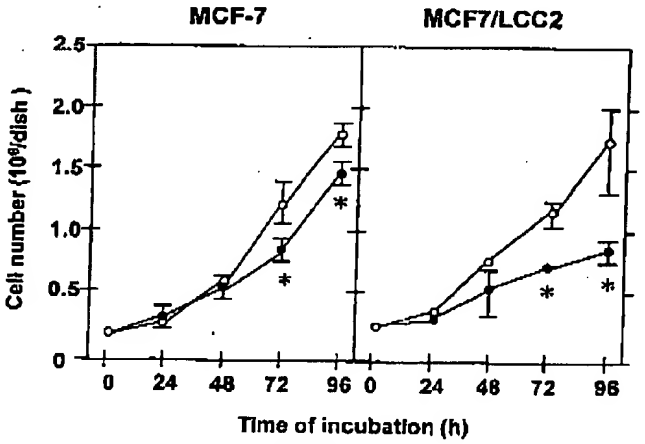

Fig. 1. Time-dependent effects of $1,25(OH)_2D_3$ on cellular proliferation. The MCF-7 and MCF7/LCC2 cells were incubated in the absence (○) or presence (●) of $1,25(OH)_2D_3$ (10^{-7} M) for 0–96 h, and *in vitro* growth was assessed. The values are representative of three independent experiments and are presented as the mean ± SD of triplicate determinations of each condition. *, values of $1,25(OH)_2D_3$-treated cells that were significantly different from the values of controls from the corresponding incubation time ($P < 0.0001$, Student-Newman-Keuls' test). In both cell lines, values obtained from both control and $1,25(OH)_2D_3$-treated cells incubated for 48–96 h were significantly higher than the values of the control cells at 0 h ($P < 0.05$, Student-Newman-Keuls' test).

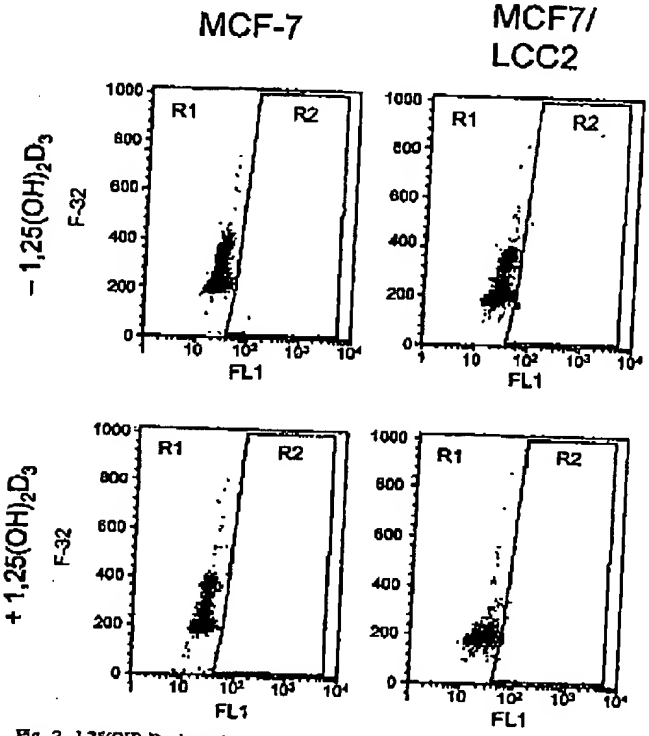

Fig. 2. $1,25(OH)_2D_3$-dependent regulation of cell cycle profiles. The MCF-7 and MCF7/LCC2 cells were incubated in the absence (−) or presence (+) of $1,25(OH)_2D_3$ (10^{-7} M) for 48 h, and cell cycle parameters were analyzed by flow cytometry. The histograms display DNA content (integrated propidium iodide fluorescence; *F-32*) versus replicative cell fractions (FITC fluorescence; *FL1*), and the profiles are representative of three independent experiments. Cells in G_1 phase, S phase, and G_2-M phase are found in channel numbers ~200, 250–350, and ~400 along the F-32 axes, respectively. Gates R2 display apoptotic cell fractions (by terminal transferase staining).

p21 and *p27*, as well as of *cdk4*, *cyclin D1*, and *p53*, all of which represent factors involved in G_1 cell cycle control (17). Of these, only *p21* was significantly up-regulated (>2-fold; *p21* mRNA: 18S rRNA ratio) by $1,25(OH)_2D_3$, but again, this was observed solely in the MCF7/LCC2 cells. However, it has also been reported that vitamin D-dependent control of the involved cell cycle factors is generally exerted at the level of the proteins (9), particularly regulated degradation of cyclin D1 (18). Hence, protein expression of the key factors was determined (Fig. 4*b*). Yet again, both cell lines showed constitutive expression of p21, p27, cdk4, and cyclin D1, but only p21 seemed to be a target for $1,25(OH)_2D_3$-dependent regulation. The induction of p21 at the protein level in the MCF7/LCC2 cells was closely identical with the corresponding regulation of its mRNA.

Finally, neither baseline nor $1,25(OH)_2D_3$-dependent expression of mRNAs for *cdk2*, the cdk4 inhibitors *p15* and *p16* (17), or the tumor suppressor *BRCA1*, which may *trans*-activate the 5′-flanking region of the *p21* gene (19), was observed in any cell line (data not shown).

Effects of Steroid Hormones and Steroid-like Factors on *com1* mRNA Expression. To examine whether cellular com1 expression may be associated with the observed growth inhibition, *com1* mRNA was measured in the MCF-7 and MCF7/LCC2 cells after treatment with $1,25(OH)_2D_3$ (10^{-7} M) for 24 h. For comparison, the cells were also incubated in the presence of 10^{-7} M each of E_2, Dex, or RA because these steroids are also known to regulate breast cancer growth (Fig. 5). In the com1-negative MCF-7 cells, expression of *com1* mRNA was below the level of detection, irrespective of the mode of treatment. Whereas E_2, Dex, or RA did not significantly alter the baseline expression of *com1* mRNA in the MCF7/LCC2 cells, a surprising up-regulation of *com1* mRNA was observed in the presence of $1,25(OH)_2D_3$.

Regulation of com1 mRNA Expression by $1,25(OH)_2D_3$. Based on the previous experiments, the MCF7/LCC2 cells were incubated in the presence of $1,25(OH)_2D_3$ (10^{-7} M) for 0–48 h, and *com1* mRNA was analyzed (Fig. 6). The expression of *com1* mRNA showed a biphasic induction (5–8-fold) with a peak level at 24 h followed by a gradual decline after 36–48 h of $1,25(OH)_2D_3$ treatment. Hence, the kinetics of this induction was much faster than the cell cycle events leading to the growth arrest of the MCF7/LCC2 cells. After 36–48 h of incubation, an up-regulation of *com1* mRNA was also observed in the control cells.

Clonal Growth of MCF-7 Cell Lines. The $1,25(OH)_2D_3$-dependent regulation of com1 seemed to be an early regulatory event in growth inhibition of the MCF7/LCC2 cells. The question of whether com1 might also be involved in long-term growth control was assessed by a colony formation assay. First, MCF7/LCC2 cells dispersed in soft agar were incubated with increasing concentrations of $1,25(OH)_2D_3$ (10^{-11} to 10^{-7} M; Fig. 7). Whereas the lower concentrations (10^{-11} to 10^{-9} M) were associated with significant stimulatory effects, half-maximal inhibition of clonal growth was observed at a concentration of $\sim 10^{-8}$ M, and complete suppression of colony formation was obtained at 10^{-7} M.

To strengthen the association between com1 expression and clonal growth of the MCF-7 cell lines, the com1-negative MCF-7 cells were stably transfected with a Dex-inducible *com1* construct, and the resulting MCF7/com1 cells were compared with the wild-type parental cells and MCF7/pMAM control transfectants with regard to colony formation in soft agar (Fig. 8). Dex at concentrations of 10^{-7} to 10^{-5} M caused inhibition (30–35%) of clonal growth of both the MCF-7 and MCF7/pMAM cells (Fig. 8*b*). In contrast, clonal growth of the MCF-7/com1 cells was inhibited by 65–75% in the presence of Dex (Fig. 8*b*). Importantly, the transfected cells expressing Dex-inducible *com1* mRNA (Fig. 8*a*) also revealed significant down-regulation (50–60%) of colony formation when compared with the MCF-7 and MCF7/pMAM cells treated with Dex (Fig. 8*b*).

DISCUSSION

In the present study we demonstrated that $1,25(OH)_2D_3$ inhibited *in vitro* growth of the com1-positive MCF7/LCC2 cells. This was associated with up-regulation of p21 and the subsequent accumulation of the hypophosphorylated form of pRB, ultimately leading to cell cycle arrest in G_1 phase. Moreover, a $1,25(OH)_2D_3$-dependent induction in *com1* mRNA expression was closely associated with the growth-inhibitory effect, suggesting that com1 may participate in the regulatory pathway involved in cellular growth inhibition.

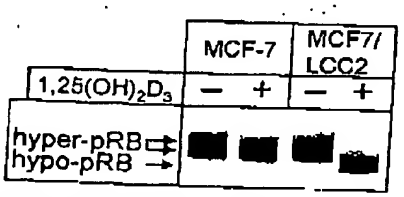

Fig. 3. $1,25(OH)_2D_3$-dependent regulation of phosphorylation pRB. The MCF-7 and MCF7/LCC2 cells were incubated in the absence (−) and presence (+) of $1,25(OH)_2D_3$ (10^{-7} M) for 48 h, and expression of hyperphosphorylated (*hyper-pRB*) and hypophosphorylated (*hypo-pRB*) forms of pRB was analyzed by Western blot hybridization. The autoradiographs are representative of two independent experiments.

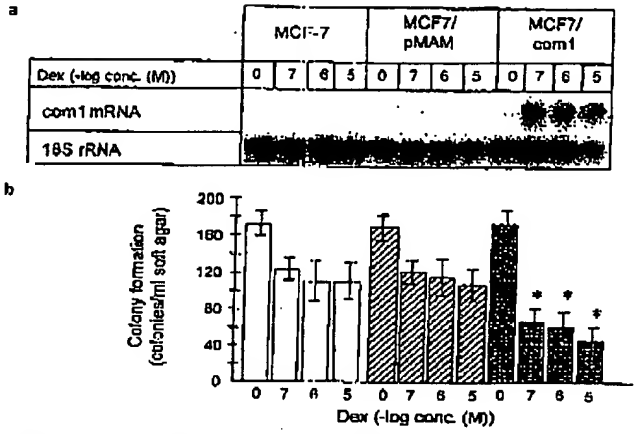

Fig. 8. com1 transfection of MCF-7 cells and effect on clonal growth. *a*, the MCF7/com1 cells represent MCF-7 cells stably transfected with a *com1* construct. The Northern blot illustrates the induction of *com1* mRNA expression in the MCF7/com1 cells by increasing concentrations (10^{-7} to 10^{-5} M) of Dex for 48 h, with the wild-type MCF-7 cells and MCF7/pMAM control transfectants used as negative controls. The autoradiographs of *com1* mRNA and 18S rRNA are representative of three independent experiments. *b*, clonal growth of the MCF7/com1 cells compared with the MCF-7 and MCF7/pMAM cells, as analyzed by a soft agar assay. Five hundred cells were seeded in 1 ml of soft agar medium containing 15% FCS, and the number of colonies was assessed after incubation in the absence (0 M) or presence of increasing concentrations (10^{-7} to 10^{-5} M) of Dex for 21 days. The values are presented as the mean ± SD of values obtained from six parallel incubations for each condition. *, values that were significantly different from the values of the MCF-7 wild-type and control MCF7/pMAM groups ($P < 0.001$, Student-Newman-Keuls' test). The MCF7/pMAM values were statistically equal to the corresponding MCF-7 values, but within all three cell lines, values obtained from Dex-treated cells were significantly lower than the control values ($P < 0.001$, Student-Newman-Keuls' test).

experimental or clinical progression of breast cancer, did not alter the expression of *com1* mRNA.

The VDR is a ligand-operated transcription factor, which principally acts in a heterodimer complex that stimulates target gene transcription via vitamin D response elements (25). Thus far, only a few primary vitamin D-responding genes have been identified (9), and *p21* is one of these (16). The possibility that the *com1* gene is transcriptionally activated by the VDR is particularly appealing but still unproven and demands comprehensive experiments on the still uncharacterized 5'-flanking region of the human *com1* gene.

The facts that the kinetics of the transient 1,25(OH)$_2$D$_3$-dependent induction of *com1* mRNA was much faster than the cell cycle events leading to growth arrest of the MCF7/LCC2 cells and that the growth rate of the com1-negative MCF-7 cells was also inhibited by 1,25(OH)$_2$D$_3$ argue against a direct involvement of com1 in cell cycle regulation. Our preliminary observation that the level of *p21* mRNA in the MCF7/com1 cell line does not apparently differ from that in the MCF-7 wild-type or MCF7/pMAM control cells (data not shown) is another argument. The increase in the level of *com1* mRNA in control MCF7/LCC2 cells after 36–48 h of incubation, which is followed by a shift toward hypophosphorylated pRB and G_1 arrest after 96 h (data not shown), illustrates the same concept. This growth retardation of the control cells is presumably due to consumption and a subsequent lack of nutrients in the medium exchanged no later than at time 0. Nevertheless, the striking inverse correlation between *com1* mRNA expression and colony formation in the MCF7/com1 cells supports the notion of some growth-regulatory effect of com1, although the suppression of clonal growth of the com1-transfected cell line was incomplete.

The gene sequence for *com1* is earlier described as the rat cDNA analogue *p8* by Mallo *et al.* (26). The authors found that pancreatic expression of p8 was strongly enhanced after stimuli that induce apoptosis (26). The kinetics of these *p8* mRNA responses seems closely comparable with our observations on 1,25(OH)$_2$D$_3$-dependent regulation of *com1* mRNA. Several reports have shown that 1,25(OH)$_2$D$_3$ and vitamin D analogues activate apoptotic death pathways in breast cancer cells (27–30). In contrast to this, our flow cytometry analysis revealed hardly any signals corresponding to apoptotic cells. However, we did not specifically examine other apoptotic features of the 1,25(OH)$_2$D$_3$-treated MCF7/LCC2 cells.

Mallo *et al.* (26) also demonstrated high levels of *p8* mRNA expression in developing and regenerating rat pancreas and liver. The authors also showed increased activity in cellular growth assays, but no alterations were seen in response to apoptotic stimuli on cellular overexpression of *p8* by transfection (26, 31). Our own initial identification of human com1 was from actively proliferating metastatic tumors (4), representing biological entities in which the relative fraction of apoptotic cells is low (32). We have also found that the expression levels of *com1* mRNA in human breast tumors are consistently and significantly higher than those in the adjacent normal breast tissue (33). Notably, the *com1* gene has a localization within chromosome 16 at position p11.2 (31), a chromosomal region occasionally amplified in primary breast tumors (34–36), which supports the assumption that com1 may function as a growth-promoting factor in the development of malignant breast tumors.

It is conceivable to assume that com1/p8 plays some regulatory role in cellular growth, although the previously published (4, 26, 31, 33) and present data are apparently conflicting. We speculate that com1 may mediate growth stimulation as well as inhibition by being recruited by preferentially stimulatory or inhibitory signals and perhaps by acting with different nuclear partners in a cell- or tissue-specific manner. To analyze the effect of com1 alone, we established the MCF7/com1 transfectants from the com1-negative MCF-7 cell line. Although we used a construct demanding the use of a potent corticosteroid analogue with an inhibitory effect on cellular proliferation, the induced MCF7/com1 cells revealed significant inhibition of colony formation in the soft agar assay compared with both the wild-type MCF-7 cells and MCF7/pMAM control transfectants treated with Dex.

In conclusion, the present study demonstrated that 1,25(OH)$_2$D$_3$ exerts highly regulated antiproliferative control of the com1-positive MCF7/LCC2 cell line. The regulatory mechanism was confirmed to involve the cell cycle inhibitor p21, and a striking association with a preceding *com1* mRNA induction was observed. The MCF7/com1 cell line established from the com1-negative MCF-7 cells seemed to mimic 1,25(OH)$_2$D$_3$-treated MCF7/LCC2 cells in a clonal growth assay. These results apparently indicate that rather than promoting growth, com1 may mediate growth inhibition of MCF-7 cell lines.

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Differential display analysis of breast carcinoma cells enriched by immunomagnetic target cell selection: gene expression profiles in bone marrow target cells.

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The red bone marrow (BM) is an important indicator organ of hematogenous micrometastatic spread of carcinomas. Characterization of biological properties specific for BM micrometastatic cells, however, is technically challenging due to the limited number of target cells usually available for the purpose. This report provides referrals to qualitative gene expression profiling of BM micrometastatic cells enriched by immunomagnetic selection. First, an experimental strategy was used to study regulatory mechanisms involved when BM micrometastatic cells colonize distant organs. The MA-11 cells, originating from BM micrometastases in a breast cancer patient clinically devoid of overt metastatic disease, were injected into immunodeficient rats. Metastatic MA-11 cells were subsequently immunoselected from the resulting in vivo lesions. The selected cell populations were compared to the injected cells by differential display analysis, and several genes possibly involved in tumor cell invasion and proliferation were confirmed as differentially expressed among the various MA-11 cell populations. A direct approach to qualitative gene expression profiling of BM micrometastatic cells was also explored. Carcinoma cells were immunoselected from BM and axillary lymph nodes obtained from breast cancer patients, and the isolated cell populations were compared by differential display analysis. Two candidate genes, identified as factors involved in cellular growth control, appeared as differentially expressed by the target cells from BM. Our study provides detailed information on how to combine an immunomagnetic selection procedure and differential display analysis to reveal gene expression profiles that may characterize BM micrometastatic cells. Copyright 2002 Wiley-Liss, Inc.

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DIFFERENTIAL DISPLAY ANALYSIS OF BREAST CARCINOMA CELLS ENRICHED BY IMMUNOMAGNETIC TARGET CELL SELECTION—GENE EXPRESSION PROFILES IN BONE MARROW TARGET CELLS

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Short title: Differential display on immunoselected cells

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Key words: bone marrow, micrometastasis, breast cancer, immunomagnetic cell preparation, differential cloning

Abbreviations: BM, bone marrow; CM, subarachnoid space 'cisterna magna'; LV, left cardiac ventricle; CNS, central nervous system; HSA/PBS, 1% human serum albumin in phosphate-buffered 0.9% NaCl (pH 7.2); AP, arbitrary primer; ARF1, ADP-ribosylation factor 1; HMP, Heart Muscle Protein; SAP18, Sin3-associated polypeptide 18 kD

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ABSTRACT

The red bone marrow (BM) is an important indicator organ of hematogenous micrometastatic spread of carcinomas. Characterization of biological properties specific for BM micrometastatic cells, however, is technically challenging due to the limited number of target cells usually available for the purpose. This report provides referrals to qualitative gene expression profiling of BM micrometastatic cells enriched by immunomagnetic selection. First, an experimental strategy was used to study regulatory mechanisms involved when BM micrometastatic cells colonize distant organs. The MA-11 cells, originating from BM micrometastases in a breast cancer patient clinically devoid of overt metastatic disease, were injected into immunodeficient rats. Metastatic MA-11 cells were subsequently immunoselected from the resulting *in vivo* lesions. The selected cell populations were compared with the injected cells by differential display analysis, and several genes possibly involved in tumor cell invasion and proliferation were confirmed as differentially expressed among the various MA-11 cell populations. A direct approach to qualitative gene expression profiling of BM micrometastatic cells was also explored. Carcinoma cells were immunoselected from BM and axillary lymph nodes obtained from breast cancer patients, and the isolated cell populations were compared by differential display analysis. Two candidate genes, identified as factors involved in cellular growth control, appeared as differentially expressed by the target cells from BM. Our study provides detailed information on how to combine an immunomagnetic selection procedure and differential display analysis to reveal gene expression profiles that may characterize BM micrometastatic cells.

INTRODUCTION

Clinical and experimental evidence suggests that epithelial tumor cells are able to disseminate to secondary organs at an early stage of primary tumor development. The red bone marrow (BM) compartment represents an important indicator organ of hematogenous micrometastatic spread of carcinomas. According to current concepts, however, disseminated tumor cells detected in the BM are not able to grow as distant metastatic lesions unless they possess certain biological characteristics, among which the ability of invasion into and proliferation within the target organ is considered highly significant (1-3).

The classical, experimental works on mechanisms of tumor metastasis demonstrated that only a small subset of cells within the parental population is capable of metastasizing (4) and that the cellular composition of secondary tumors differs from that of the primaries (5). Subsequent reports introduced the concept of dynamic heterogeneity, which argues that although the metastatic phenotype is a genetically controlled trait, it is inherently unstable (6). Indeed, cellular properties that are crucial for initiation of distant tumor growth may be obscured by the heterogeneity in both the primary tumor and the fully established metastatic lesion. Hence, to gain new insight into regulatory mechanisms of tumor metastasis one should identify molecular properties that determine the affinity of epithelial tumor cells to the BM and analyze the fluctuation of gene expression profiles that may correlate with the ability of distant growth in specific organs.

The presence of disseminated carcinoma cells in the BM or distant organs are easily evaluated by immunomagnetic selection (2, 7-12). This rapid and simple procedure enables a sensitive and specific selection of the small number of target cells

in BM aspirates and enrichment of tumor cells in tissue samples of clinical and experimental metastatic lesions.

One strategy to analyze gene expression profiles in cell populations of limited size is to use a selection technique that identifies differentially expressed genes in a qualitative manner. A solid-phase differential display protocol (13, 14) has been developed primarily for gene expression analysis of samples in which the recovery of mRNA is very low.

The aim of this study was to evaluate whether qualitative gene expression profiling of metastatic tumor cells obtained by immunomagnetic cell preparation might reveal regulatory pathways involved in the metastatic process. We compared breast carcinoma cells immunoselected from BM aspirates with target cells obtained from clinical or experimental metastases by differential display analysis, and several genes involved in stimulatory signal transduction, cell cycle regulation, and possibly angiogenesis and tumor cell invasion were identified.

MATERIALS AND METHODS

Experimental metastasis model

The MA-11 cell line was previously established from micrometastatic cells isolated by immunomagnetic selection of a BM sample taken from a breast cancer patient who was clinically devoid of metastatic disease (15). MA-11 cells in exponential growth phase were harvested and prepared for injection in athymic nude rats (Han: *rnu/rnu* Rowett), as previously reported (16). Briefly, cells in suspensions were injected into either the subarachnoid space 'cisterna magna' (CM; 2.5×10^6 cells in 30 µl per rat) or the left cardiac ventricle (LV; 2.5×10^6 cells in 200 µl per rat). The rats were sacrificed immediately upon symptoms of metastatic disease in the central nervous system (CNS). The resulting CNS lesions were dissected and mechanically dissociated in 1% human serum albumin in phosphate-buffered 0.9% NaCl, pH 7.2 (HSA/PBS) to obtain cell suspensions. These were subsequently filtered through a 70 µm cell strainer to remove remaining cellular aggregates, and the resulting single cell suspensions were adjusted to a final concentration of ~5.0×10^6 cells/ml for immunomagnetic separation. The care of animals and the experimental protocol were reviewed and approved by the National Animal Research Authority.

Human tissue specimens

Samples of red BM (~40 ml) were acquired by aspiration from the upper iliac crest of breast cancer patients at the time of surgery. After Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation (1000 *g* for 10 min), mononuclear cells from the interface layer were collected, washed, and resuspended in HSA/PBS to a final concentration of ~10^7 cells/ml for immunomagnetic separation. Tissues in excess

of what was necessary for diagnostic purposes (primary tumor and axillary lymph nodes) were obtained directly after resection. These were mechanically dissociated in HSA/PBS, and the suspensions were subsequently incubated in a mixture of 0.1 IU/ml Collagenase and 0.8 IU/ml Dispase (Boehringer Mannheim, Mannheim, Germany) for 4 min at 37°C before filtering through a 70 μm cell strainer. The resulting single cell suspensions were adjusted with HSA/PBS to a final concentration of ~10^7 cells/ml for immunomagnetic separation. Informed consent to use the tissue specimens for research purposes was obtained from the patients in advance of these procedures.

Monoclonal antibodies

The 'panhuman' antibody (17) (kindly provided by Dr. Ø. S. Bruland, The Norwegian Radium Hospital, Oslo, Norway) reveals a unique reactivity with human cells whereas rodent cells show a complete lack of reactivity (18). MOC-31 (obtained from Dr. L. de Leij, University of Groningen, Groningen, the Netherlands) is an IgG1 class antibody that binds to an epithelial cluster 2 antigen (EGP-2) on carcinoma cells (19). The antibodies were directly conjugated to superparamagnetic sheep-antimouse IgG particles (Dynabeads SAM-450; Dynal A.S., Oslo, Norway) as recommended by the manufacturer. Beads conjugated with the 'panhuman' antibody were used to isolate MA-11 cells from the experimental CNS lesions in rats, whereas beads conjugated with MOC-31 were used for immunomagnetic selection of carcinoma cells from the human tissue specimens.

Immunomagnetic cell separation

All solutions and cell preparations were kept on ice during the whole procedure to avoid nonspecific binding of immunobeads. As previously reported (10,

11) the prepared cell suspensions were transferred to 10 ml round bottom polystyrene tubes (Nunc, Naperville, IL, U.S.A.). The antibody-coated beads were added at a ratio of 2:1 to total number of cells, and the suspensions were incubated for 30 min at 4°C on a rotating mixer. The cells were subsequently diluted in HSA/PBS to a final volume of 3.0 ml and left in a magnet holder for 2 min, and the supernatants, containing unbound cells, were decanted. Of the remaining positive fractions of ~200 µl, 20 µl aliquots were examined by light microscopy for the principal presence of cells with ≥5 immunobeads bound to their surface (bead rosettes). The positive cell fractions from the experimental CNS lesions contained ~2.5×10^5 bead rosettes/sample, whereas the recovery of immunoselected target cells from the human tissue specimens showed some greater variation (see RESULTS AND DISCUSSION). The buffer volume was pipetted off the positive fractions with the tubes in the magnet holder. The remaining rosetted cells were lysed in a hypertonic buffer containing 1% sodium dodecyl sulphate (for RNA isolation when used in differential display analysis) or in TRIzol reagent (Life Technologies, Inc., Rockville, MD, U.S.A.; for RNA isolation in Northern blot analysis), and the lysates were stored at –70°C until analysis. The supernatants of the cell suspensions (negative cell fractions) were examined by light microscopy for the principal absence of cells with immunobeads bound to their surface, then pelleted by centrifugation and lysed. Quality control of positive and negative cell fractions has been documented previously (7, 8, 10).

Differential display analysis

The differential display methodology originally described (20, 21) was modified to utilize biotinylated anchored primers (5'-T_{11}VN-3') coupled to M-280

streptavidin-coated magnetic particles (Dynabeads, Dynal A.S.) for mRNA extraction (13, 14). In the present study lysates from 6 x 10^3–10^4 cells were used for this selective mRNA capture, which would give an optimal yield of extracted mRNA for the subsequent cDNA synthesis and PCR reactions (13). The first strand cDNA synthesis was carried out using Moloney murine leukemia virus Reverse Transcriptase (Life Technologies, Inc.). The cDNA generated was subsequently amplified using Taq DNA polymerase (Boehringer Mannheim), [^{35}S]dATPα (Amersham Pharmacia Biotech, Uppsala, Sweden) as label, and the anchored primers in conjunction with five different upstream arbitrary primers (APs). These were AP2 (5'-CTGATCCATG-3'), AP3 (5'-GAAACGGGAC-3'), AP4 (5'-GTGACGTAGG-3'), AP6 (5'-AGGTGACCGT-3'), and AP8 (5'-AGCCAGCGAA-3'). Thirty-five cycles of PCRs were performed, each consisting of 30 s at 94°C, 60 s at 34°C, and 120 s at 72°C. The PCR products were resolved on standard denaturing sequencing gels that were subsequently dried and subjected to autoradiography. Unique PCR fragments were identified and excised from the gels, and the eluted products were reamplified using the original set of primers and the same thermal cycling condition. The reamplified products were sequenced directly (by means of AmpliCycle Sequencing; Amersham Pharmacia Biotech) according to the manufacturer's instructions and used as probes to verify differential expression by Northern blot hybridization. The reproducibility of each step in this solid-phase differential display protocol has been documented previously (13, 14).

Northern blot RNA analysis

Four parallel samples from each experimental CNS lesions were pooled to obtain ~10^6 bead rosettes for the extraction of total RNA. Northern blot RNA analysis

was also performed on some of the negative cell fractions collected as well as on BM mononuclear cells obtained from healthy volunteers. The probes (PCR and cDNA fragments) were labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) by random priming technique, and standard Church hybridization conditions were used. A cDNA for *ADP-ribosylation factor 1* (*ARF1*) (22) was kindly provided by Dr. M. Vaughan and Dr. J. Moss at the National Institutes of Health (Bethesda, MD, U.S.A.), whereas a cDNA for *Heart Muscle Protein* (*HMP*) (23) was a generous gift from Dr. N. Tsuchida at Tokyo Medical and Dental University (Tokyo, Japan). A cDNA fragment confirmed to represent *T-plastin* (24) was cloned from a liver cDNA library (25). The IMAGE cDNA clone 201315 (GenBank accession no. R99689), corresponding to *Sin3-associated polypeptide 18 kD* (*SAP18*) (26), and a cDNA fragment (GenBank accession no. X89832) corresponding to *mitochondrial DNA for loop attachment sequence* (27) were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). Finally, to evaluate the amounts of RNA loaded, the filters were hybridized to kinase-labeled oligonucleotides specific for human 18S rRNA or a conserved sequence of 28S rRNA.

RESULTS AND DISCUSSION

Micrometastasis status has been proposed as an entry in the TNM classification system of the International Union Against Cancer—UICC as a prognostic factor for several types of solid cancers (28). In patients with primary breast cancer the presence of BM micrometastases is significantly associated with shorter survival, but is not an independent prognostic factor, as shown by short-term as well as long-term follow-up studies (29, 30). Hence, it might be specific cellular properties, rather than the mere presence of disseminated carcinoma cells in BM, that determine the clinical outcome.

Characterization of biological properties specific for BM micrometastatic cells, however, is technically challenging due to the low number of target cells usually available for the purpose. Here we demonstrate an experimental as well as a direct approach to qualitative gene expression profiling of immunoselected target cells that may functionally represent BM micrometastatic cells.

Differential display analysis of immunoselected MA-11 cell populations

The experimental strategy was by the use of the MA-11 cells injected into athymic nude rats. This cell line originated from BM micrometastases in a patient with T2N1M0 lobular breast carcinoma (15) and may therefore be considered to hold biological properties characteristic for breast carcinoma cells with propensity to form secondary tumors (16). As previously reported (16, 18), the rats injected CM showed signs of high intracranial pressure (usually hemiparesis) as a result of massive meningeal tumor growth after ~25 days, whereas the rats injected LV developed hind leg paralysis caused by spinal cord metastasis after ~40 days.

Metastatic MA-11 cells were immunoselected from the CNS lesions of the CM and LV injected rats, and the segregated tumor cell populations were compared with each other and the injected cells (MA-11 cells in culture) by differential display analysis. Differentially expressed PCR fragments were considered to represent true site-specific properties if they were present in the CM or LV cell population when absent in the two others and when not concomitantly present in rat meninges or spinal cord (*i.e.*, not evidently representing contamination from host tissues). By these criteria, five candidate fragments were identified (CM1–3, LV1–2) (Fig. 1). Their expression was evaluated by Northern blot analysis in three independent sets of experiments. The CM1 fragment appeared to be a false positive since its mRNA was below the level of detection (not shown), reflecting the difference in sensitivity between these two RNA expression assays. The LV2 fragment also turned out to represent a false positive since the 2.7 kb mRNA was not differentially expressed by the three MA-11 cell populations (Fig. 2). For the three remaining bands (LV1, CM2, CM3) differential expression, defined as ≥2.0-fold difference in the mRNA/18S rRNA level between at least two of the MA-11 cell populations, was apparently found (Fig. 2). As seen from Fig. 2*b*, the 1.9 kb LV1 mRNA showed a 2.8-fold higher level in the LV population compared with the CM population, which further showed another 3.0-fold up-regulation relative to the MA-11 cells in culture. Compared with the cultured MA-11 cells, expression of the 3.4 kb CM2 mRNA was elevated in the CM population but depressed in the LV population, so that the difference in expression levels were exactly 2.0-fold between the CM and LV populations. The 0.7 kb CM3 mRNA also showed a 2.0-fold up-regulation in the CM population compared with the two others, in which the mRNA levels were equal. For all fragments (except CM1) distinct mRNA expression was observed by the Northern blot analysis even

when expression was apparently absent in the far more sensitive differential display assay. This phenomenon has been reported, but not directly explained, also by others (31, 32). It is presumably related to the exponential nature of the PCR reaction and the probability that cDNA amplification may occasionally fail under the PCR conditions required for the differential display analysis.

Notably, a complete lack of expression by the host tissues was observed for all candidate fragments (Fig. 2*a*). This, together with the distinct mRNA expression of the candidate genes by the CM and LV populations, argues against significant contamination of rat cells in the immunomagnetic cell preparations. As previously reported (18), these preparations were also evaluated by light microscopy for host cell contamination (*i.e.*, the presence of cells with <5 immunobeads bound to their surface). This revealed a highly enriched CM population, whereas the LV population contained ~20% cells defined as contaminants.

All candidate fragments were identified as human sequences in sequence databases (Table 1). LV1 appeared to be the guanine nucleotide-binding protein ARF1, which is involved in amplification of stimulatory signal transduction (22, 33, 34). CM2 turned out to represent T-plastin. This protein participates in bundling up actin filaments (24, 35, 36), which are cellular motility structures closely associated with tumor cell invasiveness (3). Interestingly, by differential display analysis the expression level of T-plastin has also been shown to correlate with cellular resistance to DNA-damaging agents (37, 38). CM3 was identified as SAP18, a component of a nuclear complex involved in transcription repression by means of histone deacetylation (26). LV2 appeared to be the mitochondrial 'mechano-enzyme' HMP (23), also known as mitofilin (39). Finally, CM1 was identified as a clone encoding mitochondrial DNA for loop attachment sequence (27). The mRNA expression

patterns of the candidate PCR fragments were confirmed in a set of independent Northern blot experiments with the corresponding cDNAs obtained from independent sources (see MATERIALS AND METHODS).

As we have recently reported (18), the MA-11 metastasis model was used to identify a novel factor, termed com1, being up-regulated in the LV cell population. We have also found that the expression levels of *com1* in clinical breast tumors are consistently and significantly higher than in the adjacent normal breast tissue (40). com1 presumably represents a helix-turn-helix type DNA-binding protein that may mediate the response of the breast carcinoma cells to growth regulatory factors (18, 41).

Differential display analysis of immunoselected breast carcinoma cell populations

The most prominent disadvantage of the differential display procedure is the high rate of false positives, which need to be screened out by mRNA expression analysis. Unfortunately, confirmation of mRNA expression generally requires larger amounts of mRNA than it is feasible to recover from the likely number of micrometastatic cells obtained by immunoselection of BM aspirates without subsequent *ex vivo* expansion of the extracted cells. Likewise, procedures for quantitative estimation of expressed genes (42-44), the utility of which has been elegantly demonstrated (45, 46), is to date technically not sensitive enough for the purpose of characterizing directly isolated BM micrometastatic cells.

The possibilities and limitations inherent to qualitative gene expression profiling of directly isolated BM micrometastatic cells were explored using tissue specimens from two breast cancer patients who had invasive ductal carcinomas but who were clinically devoid of metastatic disease (classified as T2N1M0). A BM

aspirate and two axillary lymph nodes, one evaluated by the surgeon as positive, the other as reactive, were obtained from the first patient, whereas samples from the primary tumor, one clinically positive axillary lymph node, and BM were acquired from the other patient. These specimens were subsequently subjected to immunomagnetic cell separation, and the resulting cell populations were compared within and between the individual patients by differential display analysis (Fig. 3).

Conceptually, the overall metastatic potential may depend on a set of cellular characteristics that also include the carcinoma cells' affinity for the BM microenvironment. Hence, differentially expressed PCR fragments were considered to represent properties associated with hematogenous dissemination if they were present in the BM cell population when absent in the others, resulting in the candidate PCR fragments BM1–7 (Fig. 3). Moreover, these candidates should be concomitantly present in the BM cell population from both patients. Even though this strategy might conceal biologically relevant gene expression, it was chosen as a precaution against the risk of detecting genes expressed in contaminating cells (BM mesenchymal cells). Only two candidate fragments (BM1 and BM4) were manifested by these criteria (Fig. 3 and Table 1). BM1 appeared to be RGS2/G0S8 (GenBank accession no. L13391), a regulatory protein involved in cellular signal transduction early in the cell cycle (47, 48). BM4 turned out to represent proprotein convertase 2 (GenBank accession no. NM_002594) (49, 50), an intracellular serine proteinase involved in the processing of autocrine factors that may induce tumor growth and angiogenesis (51-53).

Expression of the RGS2/G0S8 protein is earlier demonstrated in blood mononuclear cells exclusively (54). This raises the question whether BM1 represents contamination of BM mesenchymal cells from the immunomagnetic cell preparation,

even though BM1 was detected in the isolated BM population from both patients. As we have demonstrated previously (10), the selected cell fractions were evaluated for contamination (*i.e.*, the presence of cells with <5 immunobeads bound to their surface) by light microscopy. All positive fractions (immunoselected samples from BM, positive lymph nodes, and primary tumor) contained less than 10% cells defined as contaminants (not shown). The negative cell fraction from the reactive lymph node was free of cells with ≥5 immunobeads bound to their surface, whereas the 'positive' fraction contained some few contaminating cells (not shown).

In general, a low number of immunoselected target cells was recovered from the tissue specimens. Among the ~5 x 10^7 mononuclear cells harvested from each BM aspirate (~40 ml), ~10^4 carcinoma cells were recovered, which gives a frequency of 1 tumor cell in 5 x 10^3 BM cells and which is in accordance with our earlier estimates (10). The number of target cells from the positive lymph nodes, however, differed between the two patients (6 x 10^3 and ~10^6 carcinoma cells, respectively).

Immunocytochemical detection of occult epithelial tumor cells in the BM with the use of cytokeratin antibodies supports a frequency of 1–10 tumor cells in 10^6 BM mononuclear cells (2), which obviously disputes our present findings. However, the immunomagnetic separation method increases the overall sensitivity of target cell detection by at least one order of magnitude compared with immunocytochemistry (2, 55). We have in several studies demonstrated a highly specific selection of carcinoma cells from BM mononuclear cells by the use of immunomagnetic beads coated with the MOC-31 monoclonal antibody (7, 8, 10). The apparent conflicting data obtained from immunocytochemistry *versus* the immunobead detection method may also depend on the notion that expression of cytokeratin antigens by breast carcinoma cells is occasionally lost during preparation of samples for the former analysis (56).

In the present study we tested for possible expression of BM1 and BM4 by contaminating BM mesenchymal cells. BM mononuclear cells (~5 x 10^7) were prepared from aspirates (~40 ml) obtained from ten healthy volunteers. Five of these samples were subjected to immunomagnetic cell separation with the MOC-31 antibody, but the recovery, consisting of <10^3 cells with 1–3 immunobeads bound to their surface (not shown), was far too low to process for any subsequent Northern blot analysis. The five remaining donor samples, as well as the negative cell fractions from the clinical samples, were analyzed by Northern blot hybridization. The mRNA for BM4 was below the level of detection in the samples from the healthy donors as well as in all negative cell fractions (not shown). In contrast, the samples from the healthy volunteers as well as the negative cell fractions from the patients' BM and lymph nodes displayed distinct but very low expression of BM1 mRNA (not shown). This probably represents expression of RGS2/G0S8 by hematopoietic cells (54). In these control experiments we did not evaluate the utility of applying a reverse transcriptase-PCR assay for the possible expression of BM1 and BM4 by the contaminating, weakly MOC-31 positive BM cells, which might have provided important information about our experimental protocol.

Concluding remarks

In this study we have aimed to reveal some characteristics, expressed by epithelial tumor cells colonizing the BM, that may participate in determining their ability of invasion into and proliferation within distant organs. We have provided evidence in support of expression by BM micrometastatic cells of genes involved in stimulatory signal transduction, cell cycle regulation, and possibly angiogenesis and tumor cell invasion, which are highly regulated functions in the metastatic process (3).

The present report should be considered an early exploratory study, designed to evaluate a presumable association between potentially useful biomarkers and disease characteristics (57). The potential predictive or prognostic value of these genes in clinical breast cancer will require future studies on large panels of patient materials.

The elucidation of regulatory mechanisms underlying tumor cell metastasis depends on a careful choice of methodological approaches. The immunomagnetic selection technique (2, 7-12), which allows for a selective enrichment of a limited number of target cells within a heterogeneous tissue sample, affords the opportunity to perform biological profiling of target cells that represent BM micrometastatic cells. Our study has demonstrated examples of possibilities as well as limitations inherent to this approach.

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TABLE 1 – GENE EXPRESSION PROFILES IN METASTATIC BREAST CARCINOMA CELLS

Candidate PCR fragment	Identity	GenBank accession no.	Chromosomal localization	References	Expression profile
CM1	*Mitochondrial DNA for loop attachment sequence*	X89832	—	(27)	Not expressed (false positive)
LV1	*ARF1*	NM_001658	1q42	(22, 34)	Up-regulated in CM and LV cell populations
CM2	*T-plastin*	NM_005032	X	(24, 35, 36)	Up-regulated in CM cell population
CM3	*SAP18*	NM_005870	13q12.11	(26)	Up-regulated in CM cell population
LV2	*HMP1 mitofilin*	D21094 NM_006839	21q22.3	(23, 39)	Equal expression in all MA-11 cell populations (false positive)
BM1	*RGS2/G0S8*	L13391	1q31	(47, 48)	Expressed by BM micrometastatic cells *in vivo*
BM4	*Proprotein convertase 2*	NM_002594	20p11.1-11.2	(49, 50)	Expressed by BM micrometastatic cells *in vivo*

FIGURE LEGENDS

FIGURE 1 – Differential display analysis of MA-11 cell populations. MA-11 cells in culture (originating from a T2N1M0 breast cancer patient) were injected into the CM or LV of immunodeficient rats, and metastatic MA-11 cells were subsequently immunoselected from the resulting CNS lesions. Lanes: (1) cell culture population (representing BM micrometastatic cells from the patient), (2) CM population (cells isolated from rat meningeal tumors), (3) LV population (cells isolated from rat spinal cord metastases) (4) rat meninges (negative selection control for the CM population), (5) rat spinal cord (negative selection control for the LV population). Candidate PCR fragments (CM1–3 and LV1–2) are indicated by ▪. The primers used were 5'- T_{11}VG-3' and AP6 (*a*), 5'- T_{11}VG-3' and AP8 (*b*), and 5'- T_{11}VC-3' and AP4 (*c*).

FIGURE 2 – Expression of candidate PCR fragments in the MA-11 cell populations. Lanes 1–5 are as in Figure 1 and depict data from one representative of three independent experiments. (*a*) The levels of candidate mRNAs were analyzed by Northern blot hybridization. The 18S rRNA was measured as a phenotypic control, whereas the 28S rRNA was estimated as an assay control. (*b*) PhosphoImager densitometry values of each of the candidate mRNA/18S rRNA signals are plotted, relative to the corresponding values of MA-11 cells in culture.

FIGURE 3 – Differential display analysis of breast carcinoma cell populations. Tissue specimens were collected from two individual patients (P1 and P2) with T2N1M0 breast cancer. Carcinoma cells were immunoselected from BM aspirates, clinically positive axillary lymph nodes (N+), and the primary tumor (T). The negative cell

fraction was collected from a clinically reactive axillary lymph node (N–). PCR fragments with apparent BM-specific expression (BM1–7; indicated by •) were identified by sequencing. Among these only BM1 and BM4 were detected in both P1 and P2. The primers used were 5'-T_{11}VG-3' in combination with AP2 (*a*) or AP3 (*b*).

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Clinical and cell line specific expression profiles of a human gene identified in experimental central nervous system metastases.

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Some cancers, particularly malignant melanomas and carcinomas of the breast and lung, metastasize to the central nervous system (CNS) in advanced stages. In order to develop into clinically manifest metastases, hematogenously disseminated tumor cells must respond to trophic factors within the CNS microenvironment. We have previously identified a nuclearfactor, com1, expressed in human breast carcinoma cells upon formation of experimental metastatic tumors in the CNS. In the present study distinct com1 mRNA expression was detected in cerebral metastases from patients with lung carcinomas, whereas the expression level was generally much lower in glioblastomas (primary brain tumors). In tissue specimens from normal brain and lung, as well as in glioma and lung carcinoma cell lines, com1 expression was barely detectable. One potential mechanism involved in the induction of com1 expression was indicated in the metastatic MCF7/LCC2 breast carcinoma cells. Significant increases in the level of com1 mRNA were observed upon activation of receptor tyrosine kinase signaling, which is known to operate during metastatic tumor cell proliferation within the CNS. The observations in this study strengthen the assumption that com1 may be involved in the tumor cell response to regulatory signals upon metastasis formation.

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